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(54) **Title: METHODS FOR ENHANCING PLANT DEFENSE**

(54) **Titre: PROCEDES DE RENFORCEMENT DE LA DEFENSE DE VEGETAUX**

(57) Abstract

The present invention provides methods of augmenting plant defenses, in particular the generation of active oxygen species (AOS), against predator attack that include the steps of (a) introducing a nucleic acid molecule that encodes a protein selected from the group consisting of systemin, prosystemin and polygalacturonase into plant cells; (b) regenerating plants from the plant cells treated in accordance with step (a); and (c) screening the regenerated plants to identify regenerated plants having an augmented active oxygen species response.

(57) Abrégé

La présente invention se rapporte à des procédés visant à améliorer les défenses de végétaux, et notamment à accroître la génération d'espèces à oxygène actif (AOS) face à une attaque de prédateurs. Un tel procédé consiste (a) à introduire dans les cellules végétales une molécule d'acide nucléique qui code une protéine sélectionnée du groupe constitué par une systémine, une prosystémine et une polygalacturonase; (b) à régénérer des végétaux à partir des cellules végétales traitées conformément à l'étape (a); et (c) à détecter parmi les végétaux régénérés ceux qui présentent une réponse accrue de génération d'espèces à oxygène actif.

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(71) Applicant *(for all designated States except US)*: WASHINGTON STATE UNIVERSITY RESEARCH FOUNDATION [US/US]; NE 1615 Eastgate Boulevard, Pullman, WA 99164–1802 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RYAN, Clarence, A. [US/US]; NE 1280 Cove Way, Pullman, WA 99163–4607 (US). CARDENAS, Martha, O. [CO/US]; 544 NW Sunset Drive, Pullman, WA 99163 (US).

(74) Agent: McGURL, Barry, F.; Christensen O'Connor Johnson & Kindness PLLC, Suite 2800, 1420 Fifth Avenue, Seattle, WA 98121 (US).

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(54) Title: METHODS FOR ENHANCING PLANT DEFENSE

(57) Abstract

The present invention provides methods of augmenting plant defenses, in particular the generation of active oxygen species (AOS), against predator attack that include the steps of (a) introducing a nucleic acid molecule that encodes a protein selected from the group consisting of systemin, prosystemin and polygalacturonase into plant cells; (b) regenerating plants from the plant cells treated in accordance with step (a); and (c) screening the regenerated plants to identify regenerated plants having an augmented active oxygen species response.

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Description

METHODS FOR ENHANCING PLANT DEFENSE

Related Applications

This patent application claims benefit of priority from United States Provisional Patent Application Serial Number 60/129,593, filed April 16, 1999.

Field of the Invention

This invention relates to methods of enhancing the natural defensive responses of plants against pathogens, viruses and herbivores.

Background of the Invention

Continuing efforts to identify new systemic wound responsive genes in tomato leaves, that are activated by attacking herbivores and pathogens, resulted in the recent discovery of two, new wound-inducible genes in tomato plants: a gene coding for the enzyme polygalacturonase (PG), and a gene coding for the PG regulatory β-subunit (Bergey, D.R. et al. (1999), *Proc. Natl. Acad. Sci. USA*, **96**:1756-1760 (1999)). PG has been studied in fruit, abscission zones, and pollen for many years, but it had not been previously identified in leaves. The PG enzyme can liberate pectic fragments from the plant cell wall thereby activating plant defense genes. Furthermore, PG-liberated cell wall fragments are signals that activate active oxygen species (AOS) during pathogenesis (Stennis, et al. (1998), *Plant Physiol.*, 117, 1031-1036.).

Many reports in the literature have shown that AOS is a key signaling component for inducing plant defenses against pathogens (Doke, N. et al. (1996), *Gene,* 179, 45-51; Lamb, C. and Dixon, R.A. (1997), *Annu. Rev. Plant Physiol. Mol. Biol.*, 48, 251-275; Low, P.S. and Merida, J.R. (1996), *Physiol. Plant.*, 96, 533-542; Levine, A. et al., *Cell*, 79, 583-593; Draper, J. (1997), *Trends in Plant Sci.*, 2, 162-165.). These data suggest that plants, in response to herbivore attacks, potentiate the synthesis of a known component of the signaling pathway (AOS) that is a key signaling molecule in the

biochemical pathways involved in defending plants against pathogens and viruses, and possibly against herbivores. Insects are among the leading vectors for viral infections. It appears that plants have evolved a mechanism to generate AOS during insect attacks to help protect the plant against viruses as well as pathogens that are carried by the attacking pests.

Among the earliest responses that plants mount to defend themselves against pathogens is the generation of active oxygen species (AOS), primarily O_2^- and H_2O_2, frequently called the 'oxidative burst' (Lamb, C. and Dixon, R.A. (1997), *Annu. Rev. Plant Physiol. Mol. Biol.*, 48, 251-275; Low, P.S. and Merida, J.R. (1996), *Physiol. Plant.*, 96, 533-542.). This burst occurs in two phases, a very rapid increase followed by a slower, prolonged response. AOS production has been used as one gauge of the ability of the plant to respond successfully to pathogen attacks.

The oxidative burst serves to drive crosslinking of cell wall structural elements and to trigger cell death and induction of defense proteins (Levine, A. et al., *Cell*, 79, 583-593.). AOS is generated by the activation of a NADPH oxidase that is at the cell surface (Lamb, C. and Dixon, R.A. (1997), *Annu. Rev. Plant Physiol. Mol. Biol.*, 48, 251-275) producing O_2^- that is spontaneously dismutated to the more stable active oxygen species, H_2O_2. The enzyme that is required for the synthesis of salicylic acid (SA, which is another signal that mediates plant defence responses) from benzoic acid has been shown to be activated by H_2O_2 (Draper, J. *Trends in Plant Sci.*, **2**:162-165 (1997)). An NADPH oxidase gene has been identified in plants that bears homology to the enzyme involved in the oxidative burst that accompanies damage or infection in animals (Keller, T. et al. (1998), *The Plant Cell*, 10, 255-266).

Systemin is an 18 amino acid polypeptide, isolated from the leaves of tomato plants, that induces the synthesis of numerous wound-inducible proteins, such as wound-inducible proteinase inhibitor proteins. Systemin is synthesized as part of a larger, 200 amino acid precursor protein called prosystemin. The present inventors have newly discovered that systemin induces an active oxygen species response, and that plants that overexpress prosystemin have an augmented active oxygen species response.

Summary of the Invention

In accordance with the foregoing, the present invention provides methods of augmenting plant defenses, in particular augmenting the generation of active oxygen species (AOS), against predator attack that include the steps of (a) introducing a nucleic acid molecule that encodes a protein selected from the group consisting of systemin, prosystemin and polygalacturonase into plant cells; (b) regenerating plants from the plant cells treated in accordance with step (a); and (c) screening the regenerated plants to identify regenerated plants having an augmented active oxygen species response. The

plants having an augmented AOS response can be further propagated and screened, for example to generate populations of plants (such as genetically true-breeding plant lines) having an augmented AOS response. Preferably, the nucleic acid molecules that encode systemin, prosystemin or polygalacturonase are cDNA molecules or genomic DNA molecules. Presently preferred nucleic acid molecules encoding systemin, prosystemin or polygalacturonase are cDNA molecules isolated from solanaceous plants, such as tomato and potato. Preferably, nucleic acid molecules, encoding systemin, prosystemin or polygalacturonase proteins, utilized in the practice of the present invention are under the control of a constitutive promoter, such as the CaMV 35S promoter. Preferably, the regenerated plants are screened for augmented AOS response by utilizing a colorimetric assay for AOS that is based on the oxidation of a solution of 3,3 diaminobenzidine (DAB), as set forth in Thordal-Christensen, H. et al. (1997), *Plant, J.*, 11, 1187-1194, which publication is incorporated herein by reference. The methods of the present invention can be utilized, for example, to augment the endogenous defense responses of plants, *i.e.*, to enhance the production of a plant's own chemicals and proteins, such as active oxygen species and polygalacturonase, that are involved in mounting a defense response against pests, pathogens and/or herbivores. Representative examples of plants that can be treated in accordance with the present invention include tomato, potato and bell pepper plants.

Detailed Description of the Preferred Embodiment

As used herein, the terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids or their residues. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

As used herein, the term "nucleotide" means a monomeric unit of DNA or RNA containing a sugar moiety (pentose), a phosphate and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of pentose)

and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide with the four bases of DNA being adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). Inosine ("I") is a synthetic base that can be used to substitute for any of the four, naturally-occurring bases (A, C, G or T). The four RNA bases are A,G,C and uracil ("U"). The nucleotide sequences described herein comprise a linear array of nucleotides connected by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

"Oligonucleotide" refers to short length single or double stranded sequences of deoxyribonucleotides linked via phosphodiester bonds. The oligonucleotides are chemically synthesized by known methods and purified, for example, on polyacrylamide gels.

As used herein, the term "protein useful in the practice of the present invention", or grammatical variants thereof, refers to proteins such as systemin, prosystemin and polygalacturonase, capable of augmenting one or more plant defense responses, preferably the production of polygalacturonase and/or active oxygen species.

As used herein, the term "systemin" refers to the 18 amino acid inducer of wound-inducible proteins (such as wound-inducible proteinase inhibitors) purified from tomato plant leaves and disclosed in Pearce, G. et al., Science **253**: 895-898 (1991), and to functional homologs of the systemin protein, disclosed in the foregoing Pearce et al publication, that also induce the synthesis of wound-inducible proteins.

As used herein, the term "prosystemin" refers to the 200 amino acid precursor of systemin disclosed in McGurl, B. et al., Science **255**: 1570-1573 (1992), and to functional homologs of the prosystemin protein disclosed in the foregoing McGurl et al publication.

As used herein, the term "polygalacturonase" means an enzyme that catalyzes the hydrolysis of glycosidic linkages of polymerized galacturonic acid residues. Polygalacturonase (PG) activity can be measured, for example, as described in Pressey, R., *Hort. Sci.* **21**:490-492 (1986), which publication is incorporated herein by reference.

As used herein, the term "active oxygen response" refers to the production in plants of chemically reactive oxygen species, such as O_2^- and H_2O_2, in response to cellular damage (and/or changes in cells) caused by pests, pathogens and/or herbivores. The active oxygen species can act as signalling molecules or as effector molecules that harm or kill pests, pathogens and/or herbivores that attack plants.

The term "predator", as used herein, includes herbivorous, multicellular organisms that consume plants, and microorganisms, including viruses and fungi, that infect plants.

As used herein, the term "augmenting the active oxygen species response of a plant", and grammatical equivalents thereof, means augmenting (by a quantifiable, statistically significant amount, such as by greater than a two-fold, five-fold or ten-fold increase) the active oxygen species response in plants treated in accordance with the methods of the present invention compared to control plants that were not treated in accordance with the methods of the present invention. An example of a control group of plants are plants of the same variety as the variety that was treated, in accordance with the methods of the present invention, to produce plants having augmented active oxygen species response. In order to compare a group of control plants with a group of plants treated in accordance with the methods of the present invention, both groups are preferably grown under conditions that are substantially similar or identical.

The terms "alteration", "amino acid sequence alteration", "variant" and "amino acid sequence variant" refer to protein molecules with some differences in their amino acid sequences as compared to the corresponding, native, *i.e.*, naturally-occurring, proteins. Ordinarily, the variants will possess at least about 70% homology with the corresponding native protein, and preferably, they will be at least about 80% homologous with the corresponding, native protein. The amino acid sequence variants of the proteins (such as systemin, prosystemin and polygalacturonase) useful in the practice of the present invention possess substitutions, deletions, and/or insertions at certain positions. Sequence variants of proteins may be used to attain desired enhanced or reduced enzymatic activity, modified regiochemistry or stereochemistry, or altered substrate utilization or product distribution.

Substitutional protein variants are those that have at least one amino acid residue in the native protein sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Substantial changes in the activity of the proteins useful in the practice of the present invention may be obtained by substituting an amino acid with a side chain that is significantly different in charge and/or structure from that of the native amino acid. This type of substitution would be expected to affect the structure of the polypeptide backbone and/or the charge or hydrophobicity of the molecule in the area of the substitution.

Moderate changes in the activity of the protein molecules useful in the present invention would be expected by substituting an amino acid with a side chain that is similar in charge and/or structure to that of the native molecule. This type of substitution, referred to as a conservative substitution, would not be expected to substantially alter either the structure of the polypeptide backbone or the charge or hydrophobicity of the molecule in the area of the substitution.

Insertional protein variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in the native protein molecule. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid. The insertion may be one or more amino acids. Ordinarily, the insertion will consist of one or two conservative amino acids. Amino acids similar in charge and/or structure to the amino acids adjacent to the site of insertion are defined as conservative. Insertional protein variants also include insertion of an amino acid with a charge and/or structure that is substantially different from the amino acids adjacent to the site of insertion.

Deletional variants are those where one or more amino acids in the native protein molecules have been removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the protein molecule.

The terms "DNA sequence encoding", "DNA encoding" "nucleic acid molecule encoding" and "nucleic acid encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the translated polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The terms "replicable vector" "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it another piece of DNA (the insert DNA) such as, but not limited to, a cDNA molecule. The vector is used to transport the insert DNA into a suitable host cell. The insert DNA may be derived from the host cell, or may be derived from a different cell or organism. Once in the host cell, the vector can replicate independently of or coincidental with the host chromosomal DNA, and several copies of the vector and its inserted DNA may be generated. The terms "replicable expression vector" and "expression vector" refer exclusively to vectors that contain the necessary elements that permit the expression of a polypeptide encoded by the insert DNA. Many molecules of the polypeptide encoded by the insert DNA can thus be rapidly synthesized.

The terms "transformed host cell," "transformed" and "transformation" refer to the introduction of DNA into a cell. The cell is termed a "host cell", and it may be a prokaryotic or a eukaryotic cell. Typical prokaryotic host cells include various strains of *E. coli*. Typical eukaryotic host cells are plant cells, such as tomato cells, yeast cells, insect cells or animal cells. The introduced DNA is usually in the form of a vector containing an inserted piece of DNA. The introduced DNA sequence may be from the same species as the host cell or from a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign DNA and some DNA derived from the host species.

The present invention provides methods for augmenting plant defenses, in particular the generation of active oxygen species (AOS), against predator attack. The methods each include the steps of (a) introducing a nucleic acid molecule that encodes a protein selected from the group consisting of systemin, prosystemin and polygalacturonase into plant cells; (b) regenerating plants from the plant cells treated in accordance with step (a); and (c) screening the regenerated plants to identify regenerated plants having an augmented active oxygen species response. The plants having an augmented AOS response can be further cultured and screened to generate populations of plants (such as genetically true-breeding plant lines) having an augmented AOS response.

Preferably, the nucleic acid molecules that encode systemin, prosystemin or polygalacturonase are cDNA molecules or genomic DNA molecules. Presently preferred nucleic acid molecules encoding prosystemin are prosystemin cDNA molecules isolated from solanaceous plants (*i.e.*, members of the family Solanaceae), including tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), black nightshade (*Solanum nigrum*) and bell pepper (*Capsicum annuum*), such as those described in McGurl, B. et al., *Science* 255:1570-1573 (1992) and in Constabel et al., *Plant Molecular Biology* 36:55-62 (1998), both of which publications are incorporated herein by reference, and in U.S. Patent Serial No: 5,378,819, and in U.S. Patent Serial No: 5,883,076, both of which patents are incorporated herein by reference.

Representative examples of nucleic acid molecules that encode prosystemin, and that are useful in the practice of the present invention, hybridize to the tomato prosystemin cDNA, having the sequence set forth in SEQ ID NO:1, or to the complementary sequence of the sequence set forth in SEQ ID NO:1, under conditions of 2 X SSC, 55°C (preferably 2 X SSC, 62°C), more preferably under conditions of 1 X SSC, 55°C (most preferably 1 X SSC, 62°C). Hybridization can be carried out by utilizing, for example, the technique of hybridizing radiolabelled nucleic acid probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes as set forth, for example, at pages 9.52 to 9.55 of Molecular Cloning, A Laboratory Manual (2nd edition), J. Sambrook, E.F. Fritsch and T. Maniatis eds, the cited pages of which are incorporated herein by reference.

Again by way of non-limiting example, representative examples of nucleic acid molecules that are useful in the practice of the present invention encode a prosystemin protein molecule that has at least 70% amino acid sequence identity, more preferably at least 80% amino acid sequence identity, with the tomato prosystemin protein sequence set forth in SEQ ID NO:2. Sequence identity is detemined by aligning the tomato prosystemin protein sequence set forth in SEQ ID NO:2 with another sequence and introducing gaps, if necessary, to achieve the maximum percent identity, and not considering any conservative substitutions as part of the sequence identity. Neither N- or

C- terminal extensions nor insertions shall be construed as reducing identity. No weight is given to the number or length of gaps introduced, if necessary, to achieve the maximum percent identity. Amino acid sequence identity can be determined, for example, in the following manner. The tomato prosystemin protein sequence set forth in SEQ ID NO:2 can be used to search a nucleic acid sequence database, such as the Genbank database, using the program TBLASTN version 2.0.9 (Altschul et al., Nucleic Acids Research **25:** 3389-3402 (1997)). The program is used in the ungapped mode. Default filtering is used to remove sequence identities due to regions of low complexity as defined in Wootton, J.C. and S. Federhen, Methods in Enzymology 266: 554-571 (1996).

Additional DNA molecules encoding systemin and/or prosystemin can be isolated, for example, by the polymerase chain reaction strategy described in Constabel et al., *Plant Molecular Biology* 36:55-62 (1998), which publication is incorporated herein by reference.

Any nucleic acid molecule encoding a functional polygalacturonase protein is useful in the practice of the present invention. By way of non-limiting example, nucleic acid molecules encoding a functional polygalacturonase protein useful in the practice of the present invention are disclosed in: Allen, R.L. and Lonsdale, D.M., *Plant Mol. Biol.* **20:**343-345 (1992); Brown S.M. and Crouch M.L., Plant Cell **2:**263-274 (1990) and Robert L.S. et al., *Plant Mol. Biol.* **23:**1273-1278 (1993), each of which publications is incorporated herein by reference.

Presently preferred nucleic acid molecules encoding polygalacturonase are polygalacturonase cDNA molecules isolated from solanaceous plants, such as tomato and potato. Examples of tomato cDNA molecules encoding polygalacturonase include the tomato fruit polygalacturonase cDNA molecule disclosed in Grierson et al., *Nucleic Acids Research* **14:**8595-8601 (1986) and the tomato polygalacturonase cDNA encoding an mRNA expressed during abscission reported in Kalaitzis et al., *Plant Molecular Biology* **28:**647-656 (1995), each of which publications is incorporated herein by reference. Additional, representative, DNA molecules encoding polygalacturonase useful in the practice of the present invention include the DNA sequences having the following GenBank Accession Numbers: Q02096, P35336, P05117, P35337, Q05967, P24548, P26216 and X72291, each of which nucleic acid sequences is incorporated herein by reference.

Additional nucleic acid molecules encoding polygalacturonase proteins can be isolated by using cloned polygalacturonase cDNAs or genes as hybridization probes utilizing, for example, the technique of hybridizing radiolabelled nucleic acid probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes as set forth, for example, at pages 9.52 to 9.55 of Molecular Cloning, A Laboratory Manual (2nd edition),

J. Sambrook, E.F. Fritsch and T. Maniatis eds, the cited pages of which are incorporated herein by reference. Some nucleic acid molecules encoding polygalacturonase proteins can be identified by the presence of one or more conserved amino acid sequence domains in the carboxyl terminal half of the polygalacturonase protein, such as those described at page 341 of Hadfield K.A. and Bennett A.B., *Plant Physiology*, 117:337-343 (1998), and in Lester et al., *Plant Physiol.* 105:225-231 (1994), Jenkins E.S. et al., *J. Exp. Bot.* 47:111-115 (1996), Petersen et al., *Plant Mol. Biol.*, 31:517-527 (1996) and Hadfield et al., *Plant Physiol.* 117:363-373 (1998), each of which publications is incorporated herein by reference. Representative examples of conserved amino acid sequence domains that are characteristic of some polygalacturonase proteins are: PNTDG (SEQ ID NO:3), GPGHG (SEQ ID NO:4), NTDGIH (SEQ ID NO:5), GVRIKTW (SEQ ID NO:6), GDDCVSLG (SEQ ID NO:7).

Any nucleic acid molecule encoding a functional systemin is useful in the practice of the present invention. The amino acid sequences of representative examples of functional systemin molecules are set forth in SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12. Representative examples of nucleic acid molecules that encode systemin, and that are useful in the practice of the present invention, hybridize to the tomato prosystemin cDNA, having the sequence set forth in SEQ ID NO:1, or to the complementary sequence of the sequence set forth in SEQ ID NO:1, under conditions of 2 X SSC, 55°C, more preferably under conditions of 1 X SSC, 55°C. Hybridization can be carried out by utilizing, for example, the technique of hybridizing radiolabelled nucleic acid probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes as set forth, for example, at pages 9.52 to 9.55 of Molecular Cloning, A Laboratory Manual (2nd edition), J. Sambrook, E.F. Fritsch and T. Maniatis eds, the cited pages of which are incorporated herein by reference.

Preferably, nucleic acid molecules, encoding systemin, prosystemin or polygalacturonase proteins, utilized in the practice of the present invention are under the control of a constitutive promoter, such as the CaMV 35S promoter.

Routine genetic manipulation techniques can be utilized to add a transport sequence to the amino terminal end of proteins useful in the practice of the present invention. Examples of transport sequences well known in the art are disclosed, for example, in the following publications, the cited portions of which are incorporated by reference herein: von Heijne et al., *Eur. J. Biochem.*, **180**:535-545, 1989; Stryer, *Biochemistry*, W.H. Freeman and Company, New York, NY, p. 769 [1988]. Transport sequences may be employed to direct proteins useful in the practice of the present invention to a variety of cellular or extracellular locations.

Sequence variants, produced by deletions, substitutions, mutations and/or insertions, of the proteins useful in the practice of the present invention can also be used

in the methods of the present invention. The amino acid sequence variants of the proteins useful in the practice of the present invention may be constructed by mutating the DNA sequences that encode the wild-type proteins, such as by using techniques commonly referred to as site-directed mutagenesis. Nucleic acid molecules encoding the proteins useful in the practice of the present invention can be mutated by a variety of PCR techniques well known to one of ordinary skill in the art. (See, for example, the following publications, the cited portions of which are incorporated by reference herein: "PCR Strategies", M.A. Innis, D.H. Gelfand and J.J. Sninsky, eds., 1995, Academic Press, San Diego, CA (Chapter 14); "PCR Protocols: A Guide to Methods and Applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds., Academic Press, NY (1990).

By way of non-limiting example, the two primer system utilized in the Transformer Site-Directed Mutagenesis kit from Clontech, may be employed for introducing site-directed mutants into nucleic acid molecules encoding proteins useful in the practice of the present invention. Following denaturation of the target plasmid in this system, two primers are simultaneously annealed to the plasmid; one of these primers contains the desired, site-directed mutation, the other contains a mutation at another point in the plasmid resulting in elimination of a restriction site. Second strand synthesis is then carried out, tightly linking these two mutations, and the resulting plasmids are transformed into a *mutS* strain of *E. coli*. Plasmid DNA is isolated from the transformed bacteria, restricted with the relevant restriction enzyme (thereby linearizing the unmutated plasmids), and then retransformed into *E. coli*. This system allows for generation of mutations directly in an expression plasmid, without the necessity of subcloning or generation of single-stranded phagemids. The tight linkage of the two mutations and the subsequent linearization of unmutated plasmids results in high mutation efficiency and allows minimal screening. Following synthesis of the initial restriction site primer, this method requires the use of only one new primer type per mutation site. Rather than prepare each positional mutant separately, a set of "designed degenerate" oligonucleotide primers can be synthesized in order to introduce all of the desired mutations at a given site simultaneously. Transformants can be screened by sequencing the plasmid DNA through the mutagenized region to identify and sort mutant clones. Each mutant DNA can then be fully sequenced or restricted and analyzed by electrophoresis on Mutation Detection Enhancement gel (J.T. Baker) to confirm that no other alterations in the sequence have occurred (by band shift comparison to the unmutagenized control).

Again, by way of non-limiting example, the two primer system utilized in the QuikChange™ Site-Directed Mutagenesis kit from Stratagene (La Jolla, California), may be employed for introducing site-directed mutants into nucleic acid molecules encoding

proteins useful in the practice of the present invention. Double-stranded plasmid DNA, containing the insert bearing the target mutation site, is denatured and mixed with two oligonucleotides complementary to each of the strands of the plasmid DNA at the target mutation site. The annealed oligonucleotide primers are extended using *Pfu* DNA polymerase, thereby generating a mutated plasmid containing staggered nicks. After temperature cycling, the unmutated, parental DNA template is digested with restriction enzyme *DpnI* which cleaves methylated or hemimethylated DNA, but which does not cleave unmethylated DNA. The parental, template DNA is almost always methylated or hemimethylated since most strains of *E. coli*, from which the template DNA is obtained, contain the required methylase activity. The remaining, annealed vector DNA incorporating the desired mutation(s) is transformed into *E. coli*.

In the design of a particular site directed mutagenesis experiment, it is generally desirable to first make a non-conservative substitution (e.g., Ala for Cys, His or Glu) and determine if activity is greatly impaired as a consequence. The properties of the mutagenized protein are then examined with particular attention to the kinetic parameters of K_m and k_{cat} as sensitive indicators of altered function, from which changes in binding and/or catalysis *per se* may be deduced by comparison to the native enzyme. If the residue is by this means demonstrated to be important by activity impairment, or knockout, then conservative substitutions can be made, such as Asp for Glu to alter side chain length, Ser for Cys, or Arg for His. For hydrophobic segments, it is largely size that is usefully altered, although aromatics can also be substituted for alkyl side chains. Changes in the normal product distribution can indicate which step(s) of the reaction sequence have been altered by the mutation. Modification of the hydrophobic pocket can be employed to change binding conformations for substrates and result in altered regiochemistry and/or stereochemistry.

Other site directed mutagenesis techniques may also be employed with nucleic acid molecules encoding proteins useful in the practice of the present invention. For example, restriction endonuclease digestion of DNA followed by ligation may be used to generate deletion variants of proteins useful in the practice of the present invention, as described in Section 15.3 of Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, New York, NY (1989), incorporated herein by reference. A similar strategy may be used to construct insertion variants, as described in section 15.3 of Sambrook et al., *supra*.

Oligonucleotide-directed mutagenesis may also be employed for preparing substitution variants of proteins useful in the practice of the present invention. It may also be used to conveniently prepare the deletion and insertion variants of proteins useful in the practice of the present invention. This technique is well known in the art as described by Adelman et al. (*DNA* **2**:183 [1983]); Sambrook et al., *supra*; "Current

Protocols in Molecular Biology", 1991, Wiley (NY), F.T. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.D. Seidman, J.A. Smith and K. Struhl, eds., incorporated herein by reference.

Generally, oligonucleotides of at least 25 nucleotides in length are used to insert, delete or substitute two or more nucleotides in the nucleic acid molecules encoding proteins useful in the practice of the present invention. An optimal oligonucleotide will have 12 to 15 perfectly matched nucleotides on either side of the nucleotides coding for the mutation. To mutagenize wild-type proteins useful in the practice of the present invention, the oligonucleotide is annealed to the single-stranded DNA template molecule under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of *E. coli* DNA polymerase I, is then added. This enzyme uses the oligonucleotide as a primer to complete the synthesis of the mutation-bearing strand of DNA. Thus, a heteroduplex molecule is formed such that one strand of DNA encodes the wild-type protein inserted in the vector, and the second strand of DNA encodes the mutated form of the protein inserted into the same vector. This heteroduplex molecule is then transformed into a suitable host cell.

Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions. An alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type protein DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

In the practice of the present invention, plants are genetically transformed with one or more nucleic acids encoding one or more proteins capable of augmenting one or

more plant defense responses, such as the production of polygalacturonase and/or active oxygen species. Transgenic plants can be obtained, for example, by transferring plasmids that encode a protein useful in the practice of the present invention and a selectable marker gene, *e.g.*, the kan gene encoding resistance to kanamycin, into *Agrobacterium tumifaciens* containing a helper Ti plasmid as described in Hoeckema et al., *Nature*, **303**:179-181 (1983) and culturing the *Agrobacterium* cells with leaf slices, or other tissues or cells, of the plant to be transformed as described by An et al., *Plant Physiology*, **81**:301-305 (1986). Transformation of cultured plant host cells is normally accomplished through *Agrobacterium tumifaciens*. However, other methods for introducing DNA into cells such as Polybrene (Kawai and Nishizawa, *Mol. Cell. Biol.*, **4**:1172 [1984]), protoplast fusion (Schaffner, *Proc. Natl. Acad. Sci. USA*, **77**:2163 [1980]), electroporation (Neumann et al., *EMBO J.*, **1**:841 [1982]), and direct microinjection into nuclei (Capecchi, *Cell*, **22**:479 [1980]) may also be used. Transformed plant calli may be selected through the selectable marker by growing the cells on a medium containing, e.g., kanamycin, and appropriate amounts of phytohormone such as naphthalene acetic acid and benzyladenine for callus and shoot induction. The plant cells may then be regenerated and the resulting plants transferred to soil using techniques well known to those skilled in the art. A representative protocol for the genetic transformation of potato is set forth in Kumar, A., et al., Plant J. **9**: 821-829 (1996), and a representative protocol for the genetic transformation of tomato is set forth in Filatti, J.J., et al., Bio/Technology **5**: 726-730 (1987), each of which publications are incorporated herein by reference.

A gene encoding a protein useful in the practice of the present invention can be incorporated into a plant along with a promoter, such as a constitutive promoter (such as the Cauliflower Mosaic Virus 35S promoter), or a tissue-specific promoter, or an inducible promoter. An illustrative example of a responsive promoter system that can be used in the practice of this invention is the glutathione-S-transferase (GST) system in maize. GSTs are a family of enzymes that can detoxify a number of hydrophobic electrophilic compounds that often are used as pre-emergent herbicides (Weigand et al., *Plant Molecular Biology*, **7**:235-243 [1986]). Studies have shown that the GSTs are directly involved in causing this enhanced herbicide tolerance. This action is primarily mediated through a specific 1.1 kb mRNA transcription product. In short, maize has a naturally occurring quiescent gene already present that can respond to external stimuli and that can be induced to produce a gene product. This gene has previously been identified and cloned. Thus, in one embodiment of this invention, the promoter is removed from the GST responsive gene and attached to a nucleic acid encoding a protein useful in the practice of the present invention that previously has had its native promoter removed. This engineered gene is the combination of a promoter that responds to an

external chemical stimulus and a gene responsible for successful production of a protein useful in the practice of the present invention.

In addition to the methods described above, several methods are known in the art for transferring cloned DNA into a wide variety of plant species, including gymnosperms, angiosperms, monocots and dicots (see, e.g., Glick and Thompson, eds., *Methods in Plant Molecular Biology*, CRC Press, Boca Raton, Florida [1993], incorporated by reference herein). Representative examples include electroporation-facilitated DNA uptake by protoplasts in which an electrical pulse transiently permeabilizes cell membranes, permitting the uptake of a variety of biological molecules, including recombinant DNA (Rhodes et al., *Science*, **240(4849):**204-207 [1988]); treatment of protoplasts with polyethylene glycol (Lyznik et al., *Plant Molecular Biology*, **13:**151-161 [1989]); and bombardment of cells with DNA-laden microprojectiles which are propelled by explosive force or compressed gas to penetrate the cell wall (Klein et al., *Plant Physiol.* **91:**440-444 [1989] and Boynton et al., *Science*, **240(4858):**1534-1538 [1988]). Transformation of gymnosperm species can be achieved, for example, by employing the methods set forth in Han et al, *Plant Science*, **95:**187-196 (1994), incorporated by reference herein. A method that has been applied to Rye plants (*Secale cereale*) is to directly inject plasmid DNA, including a selectable marker gene, into developing floral tillers (de la Pena et al., *Nature* **325:**274-276 (1987)). Further, plant viruses can be used as vectors to transfer genes to plant cells. Examples of plant viruses that can be used as vectors to transform plants include the Cauliflower Mosaic Virus (Brisson et al., *Nature* **310:**511-514 (1984); Additionally, plant transformation strategies and techniques are reviewed in Birch, R.G., *Ann Rev Plant Phys Plant Mol Biol*, **48:**297 (1997), and Forester et al., *Exp. Agric.*, **33:**15-33 (1997). The aforementioned publications disclosing plant transformation techniques are incorporated herein by reference, and minor variations make these technologies applicable to a broad range of plant species.

Each of these techniques has advantages and disadvantages. In each of the techniques, DNA from a plasmid is genetically engineered such that it contains not only the gene of interest, but also selectable and screenable marker genes. A selectable marker gene is used to select only those cells that have integrated copies of the plasmid (the construction is such that the gene of interest and the selectable and screenable genes are transferred as a unit). The screenable gene provides another check for the successful culturing of only those cells carrying the genes of interest. A commonly used selectable marker gene is neomycin phosphotransferase II (NPT II). This gene conveys resistance to kanamycin, a compound that can be added directly to the growth media on which the cells grow. Plant cells are normally susceptible to kanamycin and, as a result, die. The presence of the NPT II gene overcomes the effects of the kanamycin and each cell with this gene remains viable. Another selectable marker gene which can be employed in the

practice of this invention is the gene which confers resistance to the herbicide glufosinate (Basta). A screenable gene commonly used is the β-glucuronidase gene (GUS). The presence of this gene is characterized using a histochemical reaction in which a sample of putatively transformed cells is treated with a GUS assay solution. After an appropriate incubation, the cells containing the GUS gene turn blue.

The plasmid containing one or more of these genes is introduced into either plant protoplasts or callus cells by any of the previously mentioned techniques. If the marker gene is a selectable gene, only those cells that have incorporated the DNA package survive under selection with the appropriate phytotoxic agent. Once the appropriate cells are identified and propagated, plants are regenerated. Progeny from the transformed plants must be tested to ensure that the DNA package has been successfully integrated into the plant genome.

Prokaryotes may also be used as host cells for the initial cloning steps of proteins useful in the practice of the present invention. They are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants generated. Suitable prokaryotic host cells include *E. coli* K12 strain 94 (ATCC No. 31,446), *E. coli* strain W3110 (ATCC No. 27,325) *E. coli* X1776 (ATCC No. 31,537), and *E. coli* B; however many other strains of *E. coli*, such as HB101, JM101, NM522, NM538, NM539, and many other species and genera of prokaryotes including bacilli such as *Bacillus subtilis*, other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may all be used as hosts. Prokaryotic host cells or other host cells with rigid cell walls are preferably transformed using the calcium chloride method as described in section 1.82 of Sambrook et al., *supra*. Alternatively, electroporation may be used for transformation of these cells. Prokaryote transformation techniques are set forth in Dower, W.J., in Genetic Engineering, Principles and Methods, **12**:275-296, Plenum Publishing Corp., 1990; Hanahan et al., *Meth. Enzymol.*, **204**:63 (1991).

As will be apparent to those skilled in the art, any plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell may also be used to clone, express and/or manipulate nucleic acid molecules encoding proteins useful in the practice of the present invention. The vector usually has a replication site, marker genes that provide phenotypic selection in transformed cells, one or more promoters, and a polylinker region containing several restriction sites for insertion of foreign DNA. Plasmids typically used for transformation of *E. coli* include pBR322, pUC18, pUC19, pUC118, pUC119, and Bluescript M13, all of which are described in sections 1.12-1.20 of Sambrook et al., *supra*. However, many other suitable vectors are available as well. These vectors contain genes coding for ampicillin and/or

tetracycline resistance which enables cells transformed with these vectors to grow in the presence of these antibiotics.

The promoters most commonly used in prokaryotic vectors include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al. *Nature*, **375**:615 [1978]; Itakura et al., *Science*, **198**:1056 [1977]; Goeddel et al., *Nature*, **281**:544 [1979]) and a tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.*, **8**:4057 [1980]; EPO Appl. Publ. No. 36,776), and the alkaline phosphatase systems. While these are the most commonly used, other microbial promoters have been utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally into plasmid vectors (see Siebenlist et al., *Cell*, **20**:269 [1980]).

Many eukaryotic proteins normally secreted from the cell contain an endogenous secretion signal sequence as part of the amino acid sequence. Thus, proteins normally found in the cytoplasm can be targeted for secretion by linking a signal sequence to the protein. This is readily accomplished by ligating DNA encoding a signal sequence to the 5' end of the DNA encoding the protein and then expressing this fusion protein in an appropriate host cell. The DNA encoding the signal sequence may be obtained as a restriction fragment from any gene encoding a protein with a signal sequence. Thus, prokaryotic, yeast, and eukaryotic signal sequences may be used herein, depending on the type of host cell utilized to practice the invention. The DNA and amino acid sequence encoding the signal sequence portion of several eukaryotic genes including, for example, human growth hormone, proinsulin, and proalbumin are known (see Stryer, *Biochemistry* W.H. Freeman and Company, New York, NY, p. 769 [1988]), and can be used as signal sequences in appropriate eukaryotic host cells. Yeast signal sequences, as for example acid phosphatase (Arima et al., *Nuc. Acids Res.*, **11**:1657 [1983]), α–factor, alkaline phosphatase and invertase may be used to direct secretion from yeast host cells. Prokaryotic signal sequences from genes encoding, for example, LamB or OmpF (Wong et al., *Gene*, **68**:193 [1988]), MalE, PhoA, or beta-lactamase, as well as other genes, may be used to target proteins from prokaryotic cells into the culture medium.

Trafficking sequences from plants, animals and microbes can be employed in the practice of the invention to direct proteins useful in the practice of the present invention to the cytoplasm, endoplasmic reticulum, mitochondria or other cellular components, or to target the protein for export to the medium. These considerations apply to the overexpression of proteins useful in the practice of the present invention, and to direction of expression within cells or intact organisms to permit gene product function in any desired location.

The construction of suitable vectors containing DNA encoding replication sequences, regulatory sequences, phenotypic selection genes and the DNA encoding proteins useful in the practice of the present invention are prepared using standard

recombinant DNA procedures. Isolated plasmids and DNA fragments are cleaved, tailored, and ligated together in a specific order to generate the desired vectors, as is well known in the art (see, for example, Sambrook et al., *supra*).

As discussed above, variants of proteins useful in the practice of the present invention are preferably produced by means of mutation(s) that are generated using the method of site-specific mutagenesis. This method requires the synthesis and use of specific oligonucleotides that encode both the sequence of the desired mutation and a sufficient number of adjacent nucleotides to allow the oligonucleotide to stably hybridize to the DNA template.

"Digestion," "cutting," or "cleaving" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at particular locations in the DNA. These enzymes are called restriction endonucleases, and the site along the DNA sequence where each enzyme cleaves is called a restriction site. The restriction enzymes used in this invention are commercially available and are used according to the instructions supplied by the manufacturers. (See also sections 1.60-1.61 and sections 3.38-3.39 of Sambrook et al., supra.)

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

Example 1

Wound Induction of AOS in Tomato Plant Leaves

In this and the subsequent examples, active oxygen species were visually detected in the leaves of plants using, 3,3 diaminobenzidine (DAB) as substrate (Thordal-Christensen, H. et al. (1997), *Plant, J.*, 11, 1187-1194, which publication is incorporated herein by reference). Briefly, plants were excised at the base of leaves or at the base of stems with a razor blade and supplied through the cut petioles or stems with a 1 mg/ml solution of DAB, pH 3.8, for eight hours under light at 25°C. Leaves of DAB-treated plants were wounded one to three times perpendicular to the main vein by crushing with a hemostat. After wounding, the plants were continually supplied with DAB solution until the experiments were terminated by immersion of the plants or leaves in boiling ethanol (96%) for 10 minutes. This treatment decolorized the leaves except for the deep brown polymerization product produced by the reaction of DAB with active oxygen species, such as H_2O_2. After cooling, the leaves were extracted at room temperature with fresh ethanol for four hours, and the leaves were preserved at room temperature in ethanol and photographed.

Systemin (25 nM), OGA (0.5 mg/mL) and chitosan (125 µg/mL) were supplied to the excised plants or leaves in solutions of DAB as described above and incubated under light until the experiments were terminated.

DAB-treated plants were exposed to methyl jasmonate vapors for the times indicated in a closed plexiglass box containing a cotton swab on which had been placed 1 μL methyl jasmonate.

Diphenylene iodonium chloride (DPI, Sigma), an inhibitor of NADPH oxidase, was applied at a final concentration of 250 μM to excised plants previously incubated in DAB for eight hours. The plants were wounded, and were continually supplied with the DAB-DPI solution for four hours, and were then treated to visualize active oxygen species, such as H_2O_2.

In this and subsequent examples, polygalacturonase and protein assays were conducted in the following manner. At specified times after wounding, extracts from leaves were prepared for PG assays as follows. 20 g of leaves were homogenized in a Sorvall Omnimizer (Sorvall, Du Pont Company, Wilmington, Delaware) in 60 ml of ice-cold 0.1 M sodium citrate buffer, pH 6.0, containing 1 M NaCl, 4 mM ascorbic acid, 5 mM dithiothreitol (DTT), 2% polyvinylpyrrolidone (PVP), 0.1% bovine serum albumin (BSA). The extracts were filtered through four layers of cheesecloth and incubated at 4°C for three hours, and centrifuged at 10,000g for 20 minutes at 4°C. Proteins were precipitated at 4°C by the slow addition of solid ammonium sulfate to a final concentration of 80% and stirred for one hour at 4°C. The precipitates were recovered by centrifugation at 10,000g for 20 minutes at 4°C, the supernatant was discarded, the pellet was dissolved in 20 ml of 1 M NaCl, and the solution was dialyzed against 1 M NaCl at 4°C for 24 hours. Aliquots equivalent to 0.1 g fresh weight were assayed for polygalacturonase (PG) activity. Protein was analyzed with a BCA protein assay kit (Pierce).

To test the hypothesis that a wound-inducible polygalacturonase enzyme (PG) in tomato leaves may promote the generation of AOS, tomato plants were assayed for the production of AOS in leaves in response to wounding. A colorimetric assay for AOS was used that is based on the oxidation of a solution of 3,3 diaminobenzidine (DAB) (as described in Thordal-Christensen, H. et al. (1997), *Plant, J.*, 11, 1187-1194, which publication is incorporated herein by reference), that is supplied to excised plants or leaves at a 1 mg/ml concentration for about eight hours. The assay reactant for AOS, DAB, turns deep brown in the presence of active oxygen species, and both the intensity of the DAB coloration and its localization can be qualitatively assessed. The foregoing AOS assay disclosed in Thordal-Christensen, H. et al. (1997) was used to detect AOS in all of the subsequent examples.

The development of the DAB-H_2O_2 reaction product in the tomato leaves in response to wounding can be seen as early as one hour, with the color deepening for about four to six hours, then declining. The color was initially visible at the wound site, deepened in tissue surrounding the wound site, and then appeared in the major veins

throughout the plants. Unwounded leaves exhibit only low levels of PG or AOS. The time course of development of color closely matched the induction of PG in the leaves in response to wounding (see Figure 2B of Bergey, D.R. et al., *Proc. Natl. Acad. Sci. USA*, **96:** 1756-1760 (1999), which publication is incorporated herein by reference).

The synthesis of PG in tomato leaves caused by wounding has been shown to be systemic (Bergey, D.R. et al., *Proc. Natl. Acad. Sci. USA*, **96:** 1756-1760 (1999)). When young tomato plants having two leaves were wounded on one leaf and the other, unwounded, leaf was stained with 3,3 diaminobenzidine (DAB), the unwounded leaf also exhibited a strong increase in coloration in major and minor veins. Unwounded plants show a low, constitutive level of PG and AOS, but it increases substantially in response to wounding.

Example 2

Induction of AOS in Tomato Plant Leaves by Systemin and Oligogalacturonic Acid

Systemin, a signal for inducing systemic wound response proteins (including PG), and the enzymatic products of the action of PG on plant cell walls, oligogalacturonic acid (OGA), were both assayed for their abilities to activate AOS production by supplying systemin and OGA to young tomato plants through their cut stems. Systemin and oligogalacturonic acid were supplied to excised tomato plants through their cut stems by the feeding procedure described in Ryan, C.A., *Anal. Biochem.* **19:** 434-440 (1967), which publication is incorporated herein by reference. Within four hours, both systemin and the OGA fragments generated a strong AOS response in the plant leaves). AOS production was particularly evident in the vasculature of plants induced with systemin.

In order to confirm that the color developed in the leaves was due to the product of an oxidase, a specific inhibitor of NADPH oxidase, diphenylene iodonium chloride (DPI), was supplied to DAB-treated plants for an hour before wounding. No AOS, including H_2O_2, were generated within four hours after wounding, indicating that the oxidase causing the generation of H_2O_2 in response to wounding was inhibited. DPI also blocked the production of H_2O_2 induced in excised tomato leaves by systemin.

Example 3

PG Activity and AOS in Antisense Prosystemin Plants

Tomato plants genetically transformed with an antisense prosystemin gene, constitutively driven by the CaMV35S promoter, do not respond to wounding, since the prosystemin RNA is not functional, and prosystemin and systemin are not produced in their leaves. Wounding does not activate PG or AOS in antisense tomato plants (see Table 1 below), indicating that the inability of these plants to produce systemin in response to wounding blocks the wound induction of PG and AOS. Table 1 also shows PG activity and H_2O_2 production in leaves from unwounded control plants and wounded

plants from several plant families. In Table 1, the term "control" refers to an unwounded control plant, and the term "wounded" refers to the wounded leaf of a wounded plant.

TABLE 1

Polygalacturonase activity and AOS (H_2O_2) production in leaves from unwounded control plants and wounded plants from several plant families.

PLANTS	PG ACTIVITY (units)*			WOUND-INDUCIBLE
	Control	Wounded**	Fold Increase	H_2O_2
Solanaceae				
Tomato				
WT	0.74	2.38	3.2	+
Def1 Mutant	0.05	0.04	0	-
Antisense	0.05	0.03	0	-
Sense	2.07	nd	-	nd
Potato	0.20	1.60	8.0	+
Petunia	0.30	2.94	9.0	+
Tobacco	0.18	1.18	6.5	+
Pepper	0.66	1.96	3.0	+
Curcubitaceae				
Squash	0.83	1.83	2.2	+
Cucumber	0.76	1.26	1.7	+
Poaceae				
Corn	0.98	1.48	1.5	+
Barley	1.43	2.03	1.4	+
Wheat	1.50	2.00	1.3	+
Rice	1.50	2.35	1.6	+
Fabaceae				
Peas	0.87	1.67	1.9	+
Soybeans	0.72	0.80	1.1	-
Lentils	0.15	0.14	0	-
Alfalfa	0.64	0.66	0	-
Chickpea	0.42	0.47	1.1	-
Brassicaceae				
Arabidopsis	1.43	2.63	1.8	+

PLANTS	PG ACTIVITY (units)*			WOUND-INDUCIBLE
Malvaceae				
Cotton	1.47	2.20	1.5	+

* 1 unit = 0.1 DOD 520 nm/30 min at 37°C/0.1g fresh wt.

** 4-6 hours following wounding.

Example 4

PG Activity and AOS Production in Tomato Plants Constitutively Overexpressing a cDNA Encoding Tomato Prosystemin

Young transgenic plants constitutively expressing a prosystemin cDNA in its sense orientation constitutively overproduce prosystemin. See, McGurl, B. et al., *Science* 255:1570-1573 (1992). These plants release systemin into the plants in the absence of wounding, causing a constitutive expression of all of the wound-inducible defense genes. The leaves of the plants exhibit abnormally high levels of defense proteins, including PG. The plants exhibit a strong PG activity as well as a high constitutive AOS in their vascular tissues.

Example 5

AOS Production in *def1* Mutant Tomato Plants

In a mutant tomato line, called *def1*, the octadecanoid pathway is compromised because of a single mutation. The defense genes of these plants are not activated in response to wounding or systemin because of the defective gene, but the plants do synthesize defense proteins in response to methyl jasmonate, a derivative of jasmonic acid (JA), the downstream product of the octadecanoid pathway (Howe, G. et al. 1996., *The Plant Cell*, 8, 2067-2077) that activates defense genes *in vivo*, including PG. Because of the mutation, the plants are compromised in their ability to defend themselves against *Manduca sexta* larvae, an insect predator of tomato plants.

When the *def1* mutant plants were wounded, AOS was not generated (see Table 1 herein). However, methyl jasmonate, the product of the octadecanoid pathway, is able to stimulate the production of AOS in *def1* mutant plants. These results suggest that inability of the plants to synthesize AOS in response to wounding and systemin reflects their inability to synthesize JA, because of the defect in the octadecanoid pathway upstream from JA.

Example 6

Wound-Induced AOS Expression In a Range of Plant Species

As set forth in Table 1, 13 species from six plant families were shown to exhibit PG and generate AOS in leaves when wounded. The only exceptions were soybeans, chickpeas, lentils and alfalfa, all from the Fabaceae family. None of these four species exhibited PG in leaves before or after wounding, and none generated AOS when wounded. Peas, another member of the Fabaceae family, exhibited a strong wound-inducible PG, and generated AOS in response to wounding. The lack of the AOS response of the four legume species may be related to their nitrogen fixing systems, which may be sensitive to AOS, but since peas responded strongly to wounding by producing AOS, the possible relationship of AOS to nitrogen fixation remains to be resolved. Overall, Table I illustrates a one hundred percent coincidence between the presence or absence of PG and the presence or absence of AOS in leaves.

The accumulation of wound-induced AOS in the leaves of various plant species in response to wounding was measured. The plant leaves were wounded with a hemostat across the main vein and the assay described in Thordal-Christensen, H. et al. (1997), *Plant, J.*, 11, 1187-1194 was used to measure the AOS levels in the wounded leaves four hours after wounding. All of the monocot species assayed (corn, barley, wheat and rice), which are major agricultural crops worldwide, exhibited a strong PG activity in the leaves of unwounded plants, but exhibited no AOS. When wounded, they all showed an increase in PG activity and exhibited a strong generation of AOS, suggesting that the constitutive PG did not generate AOS, but the wound-inducible PG did. These results demonstrate the systemic nature of the AOS response, since AOS is generated throughout the vascular system of the leaves, and not just at the wound sites.

AOS has been shown to be a lethal antibiotic agent that can directly react with pathogens to kill them outright (Doke, N. et al. (1996), *Gene*, 179, 45-51). It is also possible that AOS can interact with the digestive system of herbivores to make the tissues unpalatable. A second role may be to activate genes for defense, as has been hypothesized for AOS in response to pathogen attacks (Doke, N. et al. (1996), *Gene*, 179, 45-51; Lamb, C. and Dixon, R.A. (1997), *Annu. Rev. Plant Physiol. Mol. Biol.*, 48, 251-275; Low, P.S. and Merida, J.R. (1996), *Physiol. Plant.*, 96, 533-542; Levine, A. et al., *Cell*, 79, 583-593; Draper, J. (1997), *Trends in Plant Sci.*, 2, 162-165). The presence of wound-inducible AOS may potentiate the plant to react to pathogens much more quickly and efficiently than unwounded plants.

The foregoing data, set forth in Examples 1 through 6, demonstrate that active oxygen species, such as hydrogen peroxide (H_2O_2), generated in response to wounding can be detected at wound sites and in distal leaf veins within one hour following wounding. The response is systemic and maximizes at about four to six hours in both

wounded and unwounded leaves, and then declines. The timing of the response corresponds with an increase in wound-inducible polygalacturonase (PG) mRNA and enzyme activity previously reported, suggesting that oligogalacturonide (OGA) fragments produced by PG are triggering the active oxygen species response. Systemin, oligogalacturonide fragments, chitosan and methyl jasmonate all induce the accumulation of active oxygen species in leaves. Tomato plants transformed with an antisense prosystemin gene produce neither PG activity nor active oxygen species in leaves in response to wounding, implicating systemin as a primary wound signal. The prosystemin antisense plants do produce both PG activity and active oxygen species when supplied with systemin, OGA, chitosan or MJ. A mutant tomato line (*def1*) compromised in the octadecanoid pathway does not exhibit PG activity or active oxygen species in response to wounding, systemin, OGA or chitosan, but does respond to MJ, indicating that the generation of active oxygen species requires a functional octadecanoid signaling pathway.

Among a total of 18 plant species from 6 families assayed, 14 species exhibited wound-inducible PG activity and the generation of active oxygen species (see Table 1 herein). Four species, all from the Fabaceae family, did not exhibit a wound-inducible PG activity and did not generate active oxygen species. The time course of induction of wound-inducible PG activity and active oxygen species in *Arabidopsis thaliana* leaves was similar to that observed in tomato leaves.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

Claims

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of augmenting the active oxygen species response of a plant comprising:

(a) introducing a nucleic acid molecule that encodes a protein selected from the group consisting of systemin, prosystemin and polygalacturonase into plant cells;

(b) regenerating plants from the plant cells treated in accordance with step (a); and

(c) screening the regenerated plants to identify regenerated plants having an augmented active oxygen species response.

2. The method of Claim 1 wherein the nucleic acid molecule encodes systemin.

3. The method of Claim 2 wherein the nucleic acid molecule encodes a systemin molecule comprising an amino acid sequence selected from the group consisting of the sequences set forth in SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12.

4. The method of Claim 1 wherein the nucleic acid encodes prosystemin.

5. The method of Claim 4 wherein the nucleic acid molecule hybridizes to the nucleic acid molecule set forth in SEQ ID NO:1, or to its antisense complement, under conditions of 2 X SSC, 55°C.

6. The method of Claim 4 wherein the nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:1.

7. The method of Claim 4 wherein the nucleic acid molecule consists of the nucleic acid sequence set forth in SEQ ID NO:1.

8. The method of Claim 4 wherein the nucleic acid molecule encodes prosystemin comprising the amino acid sequence set forth in SEQ ID NO:2.

9. The method of Claim 4 wherein the nucleic acid molecule encodes prosystemin consisting of the amino acid sequence set forth in SEQ ID NO:2.

10. The method of Claim 1 wherein the nucleic acid molecule encodes polygalacturonase.

11. The method of Claim 10 wherein the nucleic acid molecule encodes a polygalacturonase comprising an amino acid sequence motif selected from the group consisting of PNTDG (SEQ ID NO:3), GPGHG (SEQ ID NO:4), NTDGIH (SEQ ID NO:5), GVRIKTW (SEQ ID NO:6) and GDDCVSLG (SEQ ID NO:7).

12. The method of Claim 2 wherein the nucleic acid molecule hybridizes to the nucleic acid molecule set forth in SEQ ID NO:1, or to its antisense complement, under conditions of 2 X SSC, 55°C.

13. The method of Claim 1 wherein the plant cells are plant cells derived from a solanaceous plant species.

14. The method of Claim 13 wherein the plant cells are plant cells derived from a solanaceous plant species selected from the group consisting of tomato, potato and bell pepper.

SEQUENCE LISTING

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Orozco-Cardenas, Martha

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Pro Ser Gln Asp Ile Ile Lys Met Glu Gly Glu Gly Ala Leu Glu Ile
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09465

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12N 5/04, 15/09, 15/29, 15/63, 15/82; A01H 5/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/69.1, 430, 468, 419; 800/278, 286, 295, 317, 317.1, 317.2, 317.4 ; 536/23.1, 23.6, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG, STN CAS, WEST 2.0
search terms: SEQ ID NOS: 1-12, Active Oxygen Species (AOS), systemin, Polygalacturonase (PG), enhancing -plant- defense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5, 378, 819 A (RYAN et al) 03 January 1995 (03.01.95), see entire document, especially columns 2, 20-21.	1-2 ----- 3, 13-14
Y	STENNIS et al. Systemin Potentiates the Oxidative Burst in Cultured Tomato Cells. Plant Physiol. 1998, Vol. 117, pages 1031-1036, see page 1031.	1-3, 13-14
Y	WU et al. Activation of Host Defense Mechanisms by Elevated Production of H2O2 in Transgenic Plants. Plant Physiol. 1997, Vol. 115, pages 427-435, see pages 430, 432-433.	1-3, 13-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
29 JUNE 2000

Date of mailing of the international search report
04 AUG 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
MEDINA A. IBRAHIM
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09465

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERGEY et al. A Wound- and Systemin-inducedible Polygalacturonase in Tomato Leaves. Proc. Natl. Acad. Sci. USA. February 1999, Vol. 96, pages 1756-1760, see page 1756.	10-11
Y	HADFIELD et al. Polygalacturonases: Many Genes in Search of a Function. Plant Physiol. 1998, Vol. 117, pages 337-343, see page 342, last full paragraph.	10-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09465

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-3, 10-11, 13-14

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09465

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 430, 468, 419; 800/278, 286, 295, 317, 317.1, 317.2, 317.4 ; 536/23.1, 23.6, 24.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-3 and 13-14, drawn to a method of augmenting the active oxygen species response of a plant with a nucleic acid molecule encoding systemin in plants.
Group II, claim(s) 4-9 and 12, drawn to a method of augmenting AOS response of plants by transforming the plant with a nucleic acid molecule encoding prosystemin.
Group III, claim(s) 10-11, drawn to a method of augmenting AOS response of plants employing a nucleic acid molecule encoding polygalacturonase.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claimed method of transforming a plant cell with a nucleic acid molecule encoding a systemin, prosystemin or polygalacturonase in order to augment the AOS response of a plant is anticipated by Ryan et al (US 5,378,819), as set forth in the Search Report, and so does not constitute a single special technical feature which would be an advance over the prior art.

The invention of Group I, drawn to a first method, requires a nucleic acid molecule encoding systemin and a transgenic plant expressing it not required by any other group.

The invention of Group II, drawn to a second method, requires a nucleic acid molecule encoding prosystemin and a transgenic plant expressing it not required by any other group.

The invention of Group III, drawn to a third method, requires a nucleic acid molecule encoding polygalacturonase and a transgenic plant comprising it not required by any other group.